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Effect of temperature on the microbial community responsible for methane production in alkaline NamCo wetland soil

Yongcui Deng[a], Pengfei Liu[b], Ralf Conrad[b,*]

[a] College of Geography, Nanjing Normal University, Wenyuan Road 1, 210023, Nanjing, China
[b] Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Straße 10, 35043, Marburg, Germany

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ABSTRACT

The Tibetan plateau is a cold environment at high altitude and is very sensitive to global climate change. Wetlands on this plateau are among the major sources of the important greenhouse gas methane, and temperature is an important factor in controlling CH_4 production. However, it remains unknown how CH_4 production and microbial processes in such wetlands respond to climate warming. Therefore, we anaerobically incubated soil slurries at 10, 20, 30 and 45 °C for 100 days to investigate the effects of temperature on CH_4 production and microbial community structure in the wetland soil next to NamCo, an alkaline lake on the Tibetan plateau. Rates and pathways of CH_4 production were determined by measuring accumulation of CH_4 and stable isotope fractionation, respectively. The microbial community structures were investigated by Illumina sequencing of bacterial and archaeal 16S rRNA and methanogenic *mcrA* genes and/or transcripts. Increasing the temperature from 10 to 30 °C enhanced CH_4 production, but at 45 °C it almost ceased and the methanogenic precursor acetate accumulated. At lower temperatures, acetate accumulated only if acetoclastic CH_4 production was inhibited with methyl fluoride. The abundance of bacterial and archaeal 16S rRNA genes and *mcrA* genes were generally around 10^8 g^{-1} dry weight soil or higher. *Alphaproteobacteria* became increasingly abundant with time of incubation (up to 100 days) and temperature increasing from 10 to 30 °C. At 45 °C, however, *Firmicutes* became the dominant bacterial phylum. While about > 70% of CH_4 was produced from acetoclastic methanogenesis at 10–30 °C, hydrogenotrophic methanogenesis was the dominant (> 82%) pathway at 45 °C. The acetoclastic genus *Methanoseata* was the main methanogen at low (10 °C) and moderate (20 °C and 30 °C) temperatures, but the hydrogenotrophic genera *Methanoregula* and *Methanomassiliicoccus* dominated at high temperature (45 °C). *Bathyarchaeota*, with unknown function, dominated next to methanogenic *Euryarchaeota* the archaeal community, especially on DNA level. The methanogenic archaeal community composition of NamCo soil resembled that of alkaline lake sediments from the Tibetan Plateau. Structure and function of the methanogenic microbial community in alkaline wetlands and their temperature response seem to be different from those found in neutral paddy soil or acidic peatland soil.

1. Introduction

Methane (CH_4) is an important greenhouse gas with 28 times the global warming potential of CO_2 on a centennial scale (IPCC, 2013). Methane production (methanogenesis) is the final step in the anaerobic decomposition of complex organic matter involving hydrolytic and fermenting bacteria and methanogenic archaea (Zinder, 1993; Schink and Stams, 2013). Temperature is a key factor controlling the production of CH_4 in various ecosystems (Schulz et al., 1997; Fey and Conrad, 2000; Metje and Frenzel, 2005; Peng et al., 2008; Conrad et al., 2009; Tveit et al., 2015; Lu et al., 2015; Cui et al., 2015; Fu et al., 2015). Change in temperature affects many characteristics of the methanogenic microbial community, including thermodynamics and kinetics of the underlying processes, the relative contribution of hydrogenotrophic and acetoclastic pathways of CH_4 production, and the composition of the bacterial and archaeal communities involved (Conrad, 2008). Rates of production as well as emission of CH_4 from wetlands generally increase with increasing temperature exhibiting apparent activation energies of around 0.98 eV (about 95 kJ mol^{-1}) (Yvon-Durocher et al., 2014). In many methanogenic environments CH_4 production reaches a temperature optimum at about 25–35 °C and then decreases rapidly. However, some environments, for example flooded soils, can produce CH_4 also at elevated temperatures up to about 50 °C (Yao and Conrad, 2000) containing a thermophilic methanogenic

* Corresponding author.
E-mail address: conrad@mpi-marburg.mpg.de (R. Conrad).

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community (Fey et al., 2001; Wu et al., 2006; Peng et al., 2008; Lu et al., 2015; Liu et al., 2018).

Increase of temperature apparently also affects the pathway of CH_4 production. While CH_4 production is usually produced by a mixture of acetoclastic and hydrogenotrophic methanogenesis, contribution of the latter pathways was found to increase gradually with increasing temperature. The reason for such increase is thought being caused by the gradual change in thermodynamics, which favors H_2 production by syntrophic fermenting bacteria (Fey and Conrad, 2000; Glissmann et al., 2004). At sufficiently high temperatures, even syntrophic acetate oxidation becomes thermodynamically feasible (Liu and Conrad, 2010; Rui et al., 2011). As a consequence, acetoclastic methanogenesis can be replaced by syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis (Conrad et al., 2009; Liu et al., 2018). While the change in pathway and composition of the methanogenic bacterial and archaeal communities with temperature increase is usually gradual in the range of low to moderate temperatures, it can be rather abrupt at the transition to thermophilic conditions, when acetoclastic methanogenesis and acetoclastic methanogenic archaeal populations often disappear completely (Conrad et al., 2009; Liu et al., 2018).

Most of the studies of temperature effects were conducted in rice field soils (Fey et al., 2001; Peng et al., 2008; Conrad et al., 2009) or temperate lake sediments (Schulz and Conrad, 1996). A few studies focused on acidic peat soils from high latitude in cold environments (Kotsyurbenko et al., 1996; Metje and Frenzel, 2005, 2007; Tveit et al., 2015). Methanogenic pathway and community structure in peat soil can be strongly affected by the low pH values of these environments, which seems to favor hydrogenotrophic methanogenesis (Kotsyurbenko et al., 2007). Studies of temperature effects in alkaline environments, such as wetland soils on the Tibetan plateau are missing.

With a contribution of 23% to the global CH_4 emissions, natural wetlands are the largest source of CH_4 production (IPCC, 2013). Understanding how CH_4 is produced in natural wetlands is fundamental to elucidate the carbon cycle in wetlands in response to global warming. The Tibetan Plateau, known as “the roof of the world”, has an average altitude of > 4000 m. The wetlands on this Plateau account for approximately one-third of China's natural wetlands (Niu et al., 2012), and previous studies have considered these wetlands to be important sources of CH_4 (Jin et al., 1999; Ding et al., 2004). The Tibetan Plateau is called “the third pole of the earth”, it locates at low-latitude region but has cold environments, which makes it a key area for studying global changes.

The structures of methanogenic communities have been studied in various wetland soils on the Tibetan Plateau (Zhang et al., 2008; Deng et al., 2014; Cui et al., 2015; Fu et al., 2015; Tian et al., 2015; Yang et al., 2017). However, the effects of temperature on microbial CH_4 production and the methanogenic community structure have only been studied in Zoige wetland soils, which have a neutral pH (Cui et al., 2015; Fu et al., 2015). Actually, in a recent study, 8 of 13 studied wetlands on the Tibetan Plateau had soil pH > 8 (An et al., 2019), which means alkaline conditions is a feature that is characteristic for Tibetan wetland soils. Whereas Zoige wetlands are at a relatively low elevation (< 4000 m), and have neutral pH, wetlands with higher elevation (~5000 m) and higher pH remain poorly studied despite their important relevance for total CH_4 emission of the Tibetan Plateau. The structures of methanogenic communities on the Tibetan Plateau have been studied in lake sediments (Liu et al., 2013, 2016). The NamCo wetland at an altitude above 4900 m is alkaline (pH value ~9.3). It is unknown whether the effects of temperature on methanogenesis and methanogenic microbial communities are similar as in low altitude environments such as Zoige wetland soils, temperate paddy soils, lake sediments or arctic and subarctic peat, which all have neutral or acidic pH values. We hypothesized that methane production in cold climate wetland soils is less temperature sensitive than that in warm climate soils. We also hypothesized that increased temperature will change the methanogenic pathways and methanogenic community structures. We also asked whether thermophilic and alkaliphilic methanogens would exist in soils of NamCo wetland.

The Tibetan Plateau is very sensitive to global climate change and its temperature increase is approximately three times the global warming rate (Qiu, 2008). The mean annual temperature at NamCo wetland is −0.6 °C, with the maximum soil temperature of 20 °C (Wei et al., 2015). It is questioned how much more temperature change to expect in the future. The increase by 45 °C is apparently too much to be expected for the global warming. Nevertheless, we still questioned whether thermophilic methanogens would exist in soils of NamCo wetland similarly as in other soils (see above). Therefore, the objectives of this study were to evaluate the effect of low (10 °C), moderate (20 °C and 30 °C) and high (45 °C) temperature (i) on the rates of CH_4 production and acetate accumulation, (ii) the structure and dynamics of bacterial and archaeal community, and (iii) the methanogenic pathways and methanogenic community structures in alkaline peat soils of the high-altitude NamCo wetland.

2. Materials and methods

2.1. Soil sampling and anaerobic incubation

Soil cores were sampled in hollows of a wetland (30°43′N, 91°02′E, 4758 m asl.) next to NamCo (Co is the Tibetan word for lake), which is located on the Tibetan Plateau. Accordingly, this soil was named NamCo wetland soil. NamCo is an oligosaline (1.2 g salt per liter) alkaline (pH 9.3) lake. The plant community of the sampling site was dominated by *Kobresia littledalei* and *Carex moorcroftii* (Wei et al., 2015). Soil properties of NamCo wetland were reported in a previous study (Yun et al., 2014), i.e., pH 9.3, organic matter = 60.5 g kg^{-1}, total nitrogen = 3.77 g kg^{-1}. About 4 ml slurry, prepared with 1.5 g dry soil and sterile water, was transferred into 26-ml glass tubes. Then, the tubes were closed with butyl rubber stoppers and aluminum caps, flushed with N_2 for 5 times and pressurized to 0.5 bar overpressure. In parallel, tubes were treated with methyl fluoride (CH_3F, Fluorochrome Company) at an initial concentration of 1.8% (vol/vol) to specifically inhibit acetoclastic methanogenesis (Janssen and Frenzel, 1997). Then, all the tubes were incubated statically in the dark at low (10 °C), moderate (20 °C and 30 °C) or high (45 °C) temperature, respectively. The samples were incubated for 100 days and were 8 times destructively sampled. For each sampling, 24 tubes (4 temperatures × 3 replicates × 2 (with or without CH_3F)) were sacrificed. In total, 192 tubes were used for this experiment.

2.2. Chemical analyses

Gas in the headspace of tubes was sampled repeatedly during the incubation using a syringe, and analyzed for the concentrations of CH_4, CO_2, and also $\delta^{13}C$ of CH_4 and CO_2 as described before (Conrad et al., 2007). Briefly, the concentrations of CH_4 and CO_2 were measured by gas chromatography (GC). The $\delta^{13}C$ of CH_4 and CO_2 was analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). At each sampling time, the tubes were destructively sampled, and the slurries were centrifuged. The supernatant was filtered through a polytetrafluoroethylene (PTFE) membrane filter and used for analysis of acetate and propionate by high pressure liquid chromatography (HPLC). The $\delta^{13}C$ content of acetate was measured using a HPLC-C-IRMS as described previously (Conrad et al., 2007).

2.3. Calculations

The apparent isotopic fractionation factor for CO_2 conversion to CH_4 is defined as $\alpha_{app} = (\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$. The relative contribution of hydrogenotrophic methanogenesis (f_{H2}) to total CH_4 production was determined using the equation:

$f_{H2} = (\delta^{13}C_{CH4} - \delta^{13}C_{CH4-ma}) / (\delta^{13}C_{CH4-mc} - \delta^{13}C_{CH4-ma})$

Where $\delta^{13}C_{CH4}$ = $\delta^{13}C$ of total CH_4 produced; $\delta^{13}C_{CH4-mc}$ = the $\delta^{13}C$ of CH_4 produced from H_2/CO_2 (in the presence of methyl fluoride); $\delta^{13}C_{CH4-ma}$ = $\delta^{13}C$ of CH_4 produced from acetate = $\delta^{13}C_{ac\text{-}methyl} + \varepsilon_{ac,CH4}$; $\delta^{13}C_{ac\text{-}methyl}$ = $\delta^{13}C$ of the methyl group of acetate accumulated; $\varepsilon_{ac,CH4}$ = isotopic enrichment factors of acetoclastic methanogenesis. For the calculation, we assumed $\delta^{13}C_{ac\text{-}methyl}$ was 10‰ more negative than the $\delta^{13}C$ of total acetate ($\delta^{13}C_{acetate}$). We also assumed $\varepsilon_{ac,CH4} = -15$‰ for acetoclastic methanogenesis, since $\varepsilon_{ac,CH4} = -10$‰ is characteristic for *Methanosaeta* spp. (Penning et al., 2006) and $\varepsilon_{ac,CH4} = -20$‰ for *Methanosarcina* spp. (Goevert and Conrad, 2009), and since NamCo wetland soil contained both *Methanosaeta* and *Methanosarcina* species. Finally, the equation used was:

$f_{H2} = (\delta^{13}C_{CH4} - (\delta^{13}C_{Acetate} - 10–15)) / (\delta^{13}C_{CH4-CH3F} - (\delta^{13}C_{Acetate} - 10–15))$.

2.4. DNA and RNA extraction

At sampling time, triplicate tubes from each temperature were destructively sampled and the pellets of freshly centrifuged slurries were immediately frozen under liquid nitrogen and stored frozen at −80 °C for molecular analysis. DNA and RNA were extracted 3 times (including day 25, 63 and 100) from 0.4 g of frozen slurries using a modified protocol (using 2.5% sodium dodecyl sulfate (SDS) replaced CTAB in the extraction buffer) described previously (Bürgmann et al., 2003; Dumont et al., 2011). The precipitates of nucleic acids were dissolved in a final volume of 100 µl of nuclease-free water (Applied Biosystems). For RNA purification, 50 ml of the raw extract was incubated with RNase-free DNase (Qiagen) according to the manufacturer's instructions and further purified using the RNeasy MinElute Cleanup Kit (Qiagen). RNA was synthesized to single-stranded cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). DNA and cDNA samples were stored at −20 °C until the further analysis.

2.5. Quantitative PCR

For the quantification of gene copies and transcripts, DNA and cDNA preparations, respectively, were diluted by 40:1 and 10:1. Quantification of bacterial 16S rRNA, archaeal 16S rRNA and *mcrA* genes was performed in an iCycler instrument (Bio-Rad). Primers of Ba519f/Ba907r (Lane, 1991), A364aF/A934b (Burggraf et al., 1997; Großkopf et al., 1998) and mlas-mod-F/*mcrA*-rev-R (Angel et al., 2012) were used for quantification of bacterial 16S rRNA, archaeal 16S rRNA and *mcrA* gene, respectively. The thermal cycles and assay protocols were described previously (Pratscher, 2010; Angel et al., 2012).

2.6. Amplicon sequencing

Barcoded PCR amplicons were prepared by using a two-step PCR approach as described previously (Herbold et al., 2015). PCR amplification was carried out using the bacterial primer set 515F–Y/926R (Caporaso et al., 2012; Parada et al., 2016), the archaeal primer set U519F/Arch806R (Porat et al., 2010) and the methanogenic primer set mlas-mod-F/*mcrA*-rev-R (Angel et al., 2012). In a first PCR, target genes were amplified with diagnostic primers synthesized with a 16-bp head sequence 5′ -GCTATGCGCGAGCTGC-3′ at the 5′ end. In a second PCR, products were amplified with primers that consist of the 16-bp head sequence and include at the 5′ end a library-specific 8-bp barcode at the 5’ end. The barcode information for each sample is listed in Table S1. Each first PCR reaction (50 µl volumes) consisted of (5 µl 10 × AccuPrime PCR Buffer II (Life Technologies), 1 µl (10 µM) of each primer (1.5 µl for *mcrA* gene), 1 µl of Taq AccuPrime (Life Technologies), 0.5 µl BSA, 2 µl of template and sterile water. PCRs for 16S rRNA gene were performed on a Bio-Rad instrument with the following cycling conditions: initial denaturation (94 °C, 3 min), followed by 28 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30s) and elongation (68 °C, 1 min), and final extension (68 °C, 10 min). For *mcrA* gene, 38 cycles and 52 °C annealing temperature were performed in the first-step PCR as described previously. The first PCR reaction was screened by gel electrophoresis and purified with Agencourt AMPure beads (Beckman Coulter Genomics). For the second PCR, each first PCR reaction (50 µl volumes) consisted of (5 µl 10 × AccuPrime PCR Buffer II (Life Technologies), 2.5 µl (10 µM) of each primer, 1 µl of Taq AccuPrime (Life Technologies), 0.5 µl BSA, 4 µl of first PCR product and sterile water. PCRs were performed on a Bio-Rad instrument with the following cycling conditions: initial denaturation (94 °C, 3 min), followed by 10–17 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s) and elongation (68 °C, 1 min), and final extension (68 °C, 10 min). The barcoded amplicons in the second step PCR products were also screened by gel electrophoresis, purified with Agencourt AMPure beads (Beckman Coulter Genomics) and quantified using the Qubit dsDNA BR Assay Kit (Life Technologies) and a Qubit 2.0 Fluorometer system (Life Technologies) to have even proportions of each sample. The library of 16S rRNA genes was sequenced on an Illumina Hiseq 2000 system using 2 × 250 cycle combination mode by Max Planck-Genome-Centre (Cologne, Germany). The library of *mcrA* genes was sequenced on an Illumina Miseq system using 2 × 300 cycle combination mode by Microsynth (Balgach, Switzerland).

2.7. Sequences analysis

All 16S rRNA and *mcrA* gene sequences were processed using tools from programs USEARCH v8.1.1861 (Edgar, 2010), QIIME 1.9.1 (Caporaso et al., 2010), Mothur v.1.33.2 (Schloss et al., 2009) and ARB 5.5 (Ludwig et al., 2004). Firstly, paired-end reads were merged using -fastq_mergepairs command with default setting in USEARCH. The fastq_filter command was used to discard the sequences with a maximum expected error greater than 1. The -derep_fulength_command was used to remove singletons. The -sizeout command was also carried out before the operational taxonomic units (OTU) cluster. Species level 16S rRNA gene OTUs were obtained using -cluster_otus at 97% sequence similarity with the chimera detection in USEARCH. Taxonomic information of OTUs was obtained using the Ribosomal Database Project (RDP) Classifier against the SILVA 123 SSU Ref database (Pruesse et al., 2007) at a confidence level of 80% in QIIME.

Operational taxonomic units (OTU) of the *mcrA* gene were first assigned with 98% similarity in USEARCH using -cluster_otus command. Chimeras were filtered during the OTU clustering. All the OTU represented sequences were aligned to the *mcrA* reference sequence alignment in Mothur (Schloss et al., 2009). The aligned reference sequences were imported into ARB (Ludwig et al., 2004). The nucleotide sequences were translated into amino acid sequences. Sequences with translation mistakes or frameshifts were manually removed before further analysis. Distance matrix was calculated based on the nucleotide sequences, and exported from ARB into Mothur. Furthermore, new *mcrA* OTUs at approximate species-level were obtained based on 0.84 nucleotide distance (Yang et al., 2014) in Mothur. Subsequently, representative sequences of these *mcrA* OTUs were imported into ARB for taxonomic classification by the construction of phylogenetic trees using a previously established *mcrA* database (Angel et al., 2012). Sequence data are available in the NCBI Sequence Read Archive under study accession number SRP153754.

2.8. Statistical analyses

A heatmap representing the relative abundance of bacterial OTUs was constructed using R. Hellinger transformation of the OTU counts, and Principal components analysis (PCA) was performed. In order to select the OTUs representing most of the differences between samples, OTUs with highest loadings of PC1, PC2 and PC3 were chosen to

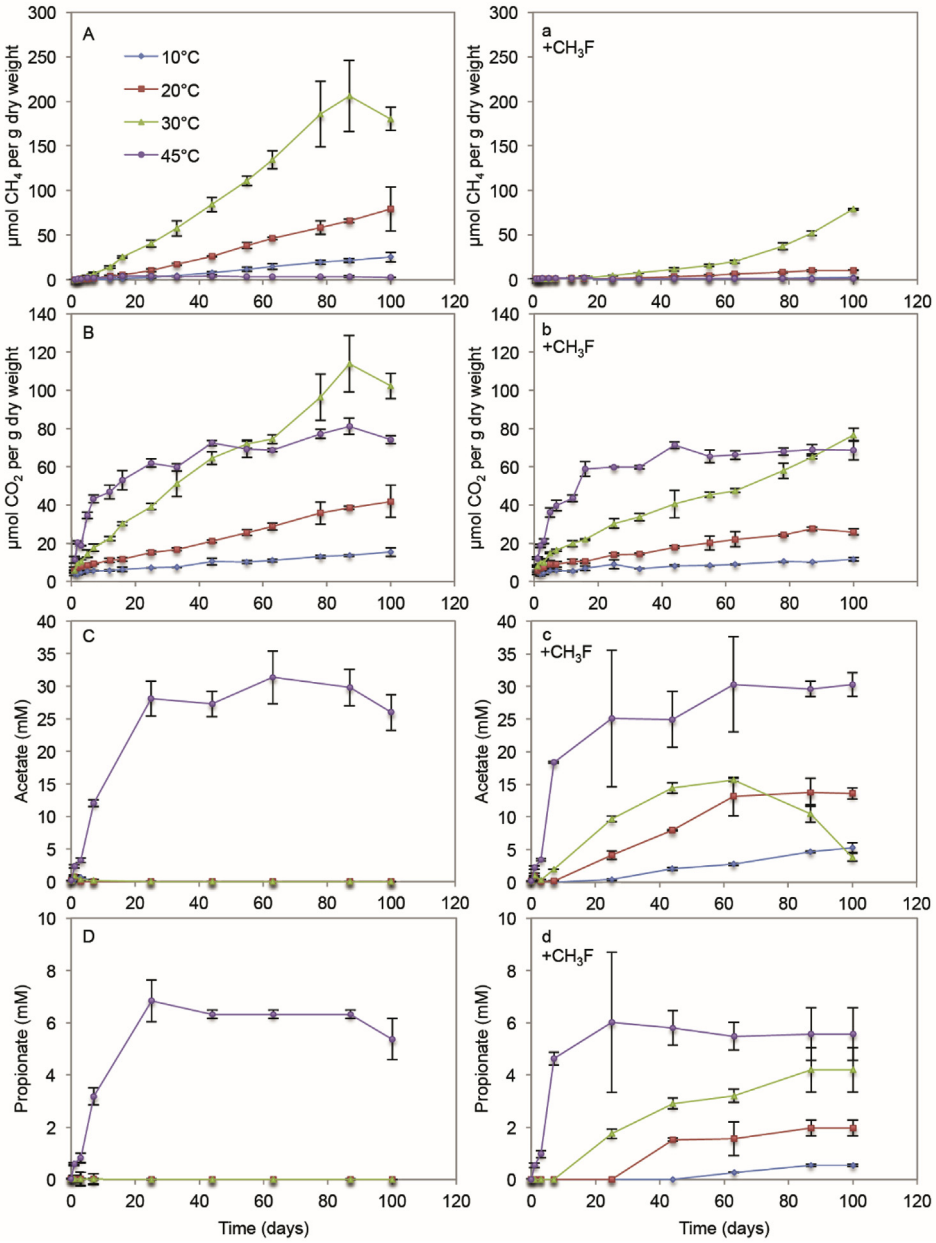

Fig. 1. Effects of temperature (10, 20, 30 and 45 °C) on CH_4 (A and a), CO_2 (B and b), acetate (C and c) and propionate (D and d) production in wetland soils without inhibiter CH_3F (A, B, C and D) and with the addition of inhibitor CH_3F (a, b, c and d). Error bars indicate standard deviations of three replicates.

construct the heatmap. The OTU abundances were converted to percentage of reads for each sample and Manhattan distances were calculated, and the heatmap constructed using the heatmap.2 function in gplots. Canonical Analysis of Principal Coordinates (CAP, Anderson and Willis, 2003), also called Distance-based redundancy analysis (db-RDA), based on distance of microbial community structures was generated using the capscale function in Vegan package of R (Oksanen et al., 2007). Non-metric multidimensional scaling (NMDS) plots based on Bray–Curtis distance of microbial community structure were generated in the R package phyloseq 1.14.0 (McMurdie and Holmes, 2013).

3. Results

3.1. CH_4, CO_2, acetate and propionate process data

Wetland soils from NamCo were incubated at four different temperatures (10, 20, 30 and 45 °C, Fig. 1). The increase of temperature from 10 to 30 °C largely enhanced the rate of CH_4 production (Fig. 1A). The rates of CH_4 production were determined by linear regression during their linear accumulation. Soils incubated in 10, 20 and 30 °C produced CH_4 at a constant rate of 0.24, 0.75, and 2.12 μmol (g dry weight)$^{-1}$ day^{-1}, respectively. The apparent activation energy at this temperature range was 77.7 kJ mol^{-1}. At high temperature (45 °C), CH_4 production was almost zero. The addition of the inhibitor CH_3F resulted in strong inhibition of CH_4 production (Fig. 1a).

Production of CO_2 occurred without lag phase and increased with increasing temperature (Fig. 1B). The production of CO_2 was linear with incubation time at low and moderate temperatures (10–30 °C). At 45 °C, however, it was nonlinear and eventually slowed down (Fig. 1B). Except at 45 °C, the addition of CH_3F significantly inhibited the CO_2 production rates (Fig. 1b).

The major fermentation intermediates acetate and propionate were barely detectable at temperatures of 10–30 °C (Fig. 1C and D), and accumulated only when CH_3F was added (Fig. 1c, d). Owing to the

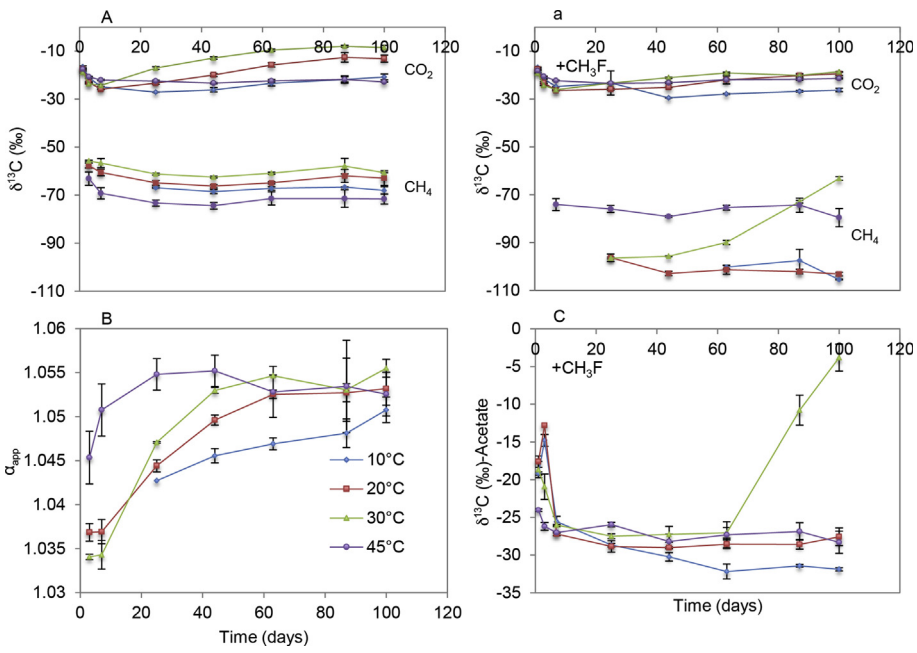

Fig. 2. Evolution of $\delta^{13}C$ (‰) values of accumulated CH_4 and CO_2 with and without CH_3F inhibitor (A and a), apparent fractionation factors α_{app}(B), and $\delta^{13}C$ (‰) values of accumulated acetate with CH_3F inhibitor (C) at different incubation temperatures (10, 20, 30 and 45 °C). Error bars indicate standard deviations of three replicates.

gradual consumption of CH_3F, especially at 30 °C (Fig. S1), the accumulated acetate decreased and CH_4 increased in the inhibited samples after about 60 days of incubation (Fig. 1a, c). At 45 °C, both acetate and propionate accumulated reaching maximum concentrations of 31 mM and 6.8 mM, respectively (Fig. 1C and D). Similar values were reached in the presence of CH_3F (Fig. 1c, d). In contrast to the other temperatures, incubation at 45 °C also resulted in a decrease of the pH below pH 7 after 25 days of incubation (Fig. S2).

3.2. $\delta^{13}C$ values of CH_4, CO_2 and acetate

The $\delta^{13}C$ values of the produced CH_4 was generally lower in the presence than in the absence of CH_3F (Fig. 2A, a), while $\delta^{13}C$ values of CO_2 were not much affected (Fig. 2A). Without CH_3F, $\delta^{13}C$ values of CH_4 and CO_2 both increased with increasing incubation temperature (10, 20 and 30 °C). The apparent fractionation factors (α_{app}) were in a range of 1.034–1.058. In general, α_{app} values increased with temperature increasing from 10 to 30 °C (Fig. 2B). At 45 °C, only little CH_4 accumulated. This CH_4 had a quite low $\delta^{13}C$ value, resulting in a high α_{app} value (Fig. 2A and B).

Because of the very low concentrations of acetate in the non-inhibited samples, their $\delta^{13}C$ values could not be measured. The $\delta^{13}C$ values of total acetate in the inhibited samples decreased from ~-15‰ to ~ -30‰ during the 100 days incubation time, the lowest values being found at 10 °C (Fig. 2C). The $\delta^{13}C$ values of total acetate at 30 °C increased after about 60 days of incubation, indicating that acetate consumption was no longer inhibited by CH_3F.

3.3. Percentage of hydrogenotrophic methanogenesis

The percentage of CH_4 produced by hydrogenotrophic methanogenesis (f_{H2}) at 10, 20 and 30 °C was similar (f_{H2} ~23%) and did not change with incubation time (Fig. 3). At 45 °C, however, CH_4 was mainly produced by hydrogenotrophic methanogenesis (f_{H2} ~82%), but note the low amounts of CH_4 produced at this temperature (Fig. 1A).

3.4. Abundance of bacteria, archaea and methanogens

Copy numbers of bacterial (Fig. 4A) and archaeal (Fig. 4C) 16S rRNA genes and *mcrA* genes (characteristic for methanogens) (Fig. 4E)

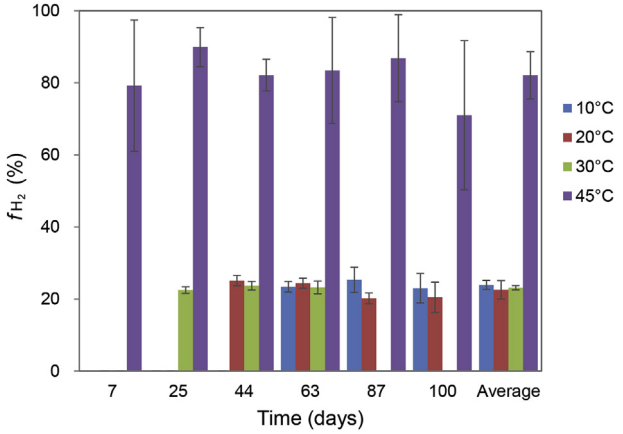

Fig. 3. Percentage of hydrogenotrophic methanogenesis. Error bars indicate standard deviations of three replicates.

were not much different between the different incubation temperatures, except incubations at 45 °C, which exhibited the lowest numbers. In general, copy numbers were highest for Bacteria, followed by Archaea and methanogens. However, even at 45 °C copy numbers of *mcrA* were still higher than 10^7 g^{-1} dry weight soil (Fig. 4E). Copy numbers of 16S rRNA transcripts decreased with incubation time and increasing temperature (Fig. 4B, D).

3.5. Bacterial community composition

The bacterial community consisted mainly of the phyla *Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi, Actinobacteria, Acidobacteria*, and *Ignavibacteriae* (Fig. 5A). From DNA data, at 10, 20 and 30 °C, the composition of the bacterial community was relatively stable during the incubation period, and temperature apparently had no effect on the bacterial community structure. *Proteobacteria* was the predominant phylum accounting for 35% of the total Bacteria. However, incubation at 45 °C resulted in a completely different bacterial community composition with *Firmicutes* becoming the predominant phylum accounting for 41% of the total bacteria (Fig. 5A).

In contrast to DNA, the RNA data exhibited greater dynamics (Fig. 5A), with the relative abundance of *Proteobacteria* increasing with

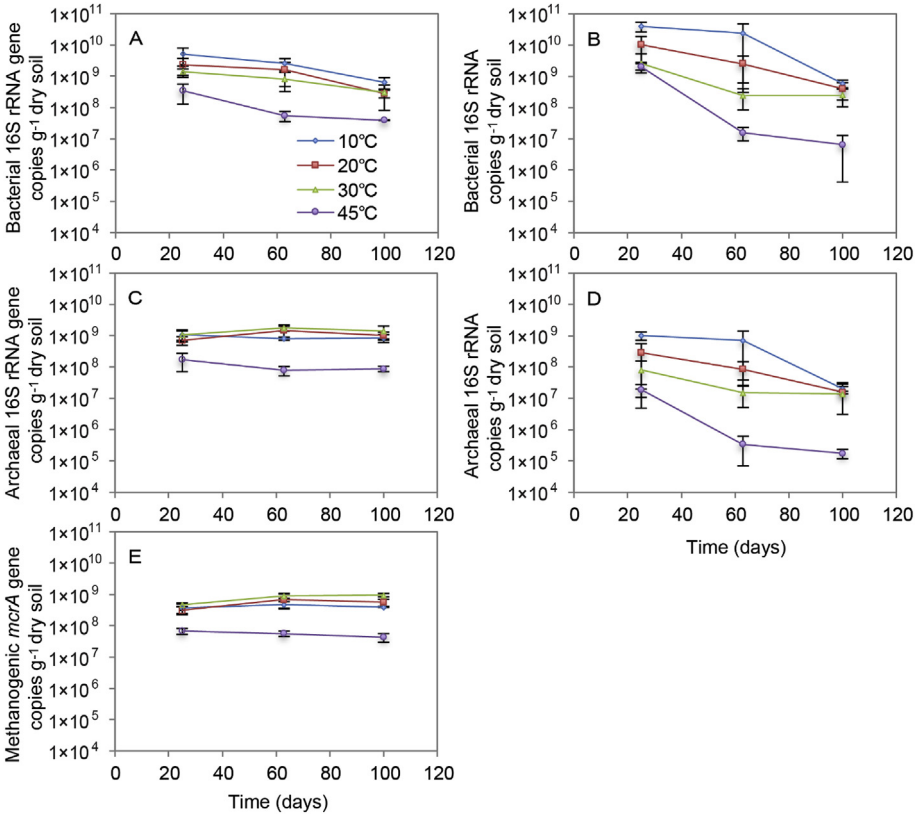

Fig. 4. Copy numbers of 16S rRNA bacterial genes of DNA or RNA (A/B), 16S rRNA archaeal genes of DNA or RNA (C/D) and *mcrA* gene (E) in soils incubated at 10, 20, 30 and 45 °C temperatures. Error bars indicate standard deviations of three replicates.

incubation time (25, 63, and 100 days) and incubation temperature (10, 20, and 30 °C). Especially the relative abundance of *Alphaproteobacteria* increased from 16% to 30%. By contrast, the relative proportion of *Bacteroidetes* decreased with the increase of temperature (10, 20, and 30 °C). Incubation at 45 °C again resulted in the dominance of *Firmicutes*, which only slightly decreased during the incubation time being replaced by *Gammaproteobacteria* (Fig. 5A).

Heatmap analysis was used to show the effect of temperature and incubation time on the composition of bacterial community (Fig. 6). Again, the bacterial community composition was significantly different at 45 °C than at the other temperatures. In addition, the bacterial community composition from DNA was different to that from RNA. At 45 °C, the phylum *Firmicutes* was mainly represented by the order *Clostridiales*, but the order *Thermoanaerobacterales* was also relatively abundant in both DNA and RNA (Fig. 6). OTU_1 belonging to *Rhodospirillales* in the class *Alphaproteobacteria* was the only OTU that was highly abundant in all the different samples (Fig. 6). Other OTUs of *Rhodospirillales* exhibited greater dynamics among the different samples. *Rhodospirillaceae* was the most abundant bacterial family. CAP analysis based on the major bacterial families showed that the relative abundance of *Rhodospirillaceae* increased with increasing incubation time and temperature from 10 to 30 °C (Fig. S3A). This increase was more obvious from RNA (Fig. S3B) than DNA (Fig. S3A). The heatmap (Fig. 6) showed that the composition of the bacterial community at 30 °C changed after about 60 days of incubation, as these samples clustered separately from the other 30 °C samples. Consistent with the heatmap analysis, NMDS analysis also showed a clear separation of the bacterial community composition on the level of RNA, and also between samples incubated at 45 °C and other temperatures (Fig. S4A).

3.6. Archaeal community composition

The main phyla of the Archaea included *Bathyarchaeota, Euryarchaeota, Thaumarchaeota, Lokiarchaeota*, and *Woesearchaeota* (Fig. 5B). From DNA data, *Bathyarchaeota* (relative abundance about 56%) was the dominant phylum at all temperatures and time points. However, from the RNA data, the relative abundance of *Euryarchaeota* was significantly larger than that of *Bathyarchaeota*, except at 45 °C, where *Bathyarchaeota* still dominated. From DNA data, *Woesearchaeota* accounted for 4% of total archaea in 10–30 °C incubations but accounted for only < 0.1% at 45 °C, and generally exhibited a low abundance (> 1%) on RNA level. Canonical methanogens belong to *Euryarchaeota*. The genus *Methanoseata* (now named *Methanothrix*; Garrity et al., 2011) was the most abundant and active methanogenic archaeon. The genus *Methanosarcina* and an unclassified cluster within the class *Methanomicrobia* were also active. Besides them, *Methanomassiliicoccus* was also detected as active archaeon (Fig. S5). CAP analysis showed the relative abundance of the family *Methanoseataceae* (recently proposed *Methanotrichaceae*; Oren, 2014) increased on DNA level with increasing incubation time and temperature from 10 to 30 °C (Fig. S3C), but on RNA level increased only with incubation time (Fig. S3D). NMDS analysis showed separation of the archaeal community compositions between samples incubated at different days and temperatures on DNA level (Fig. S4B).

3.7. Methanogenic community composition

Analysis of composition of the *mcrA* gene, which is characteristic for methanogens, confirmed that the acetoclastic genus *Methanoseata* (OTU_1) was the dominant methanogen. It accounted for an increasing percentage (50–75%) with increasing incubation time and increasing temperatures from 10 to 30 °C (Fig. 5C and S3C). In contrast, the relative abundance of the genus *Methanosarcina* decreased (Fig. 5C), indicating a functional replacement of the *Methanosarcina* by *Methanosaeta*. The genera *Methanoregula* and *Methanomassiliicoccus* were also found in low and moderate temperature. However, at high temperature

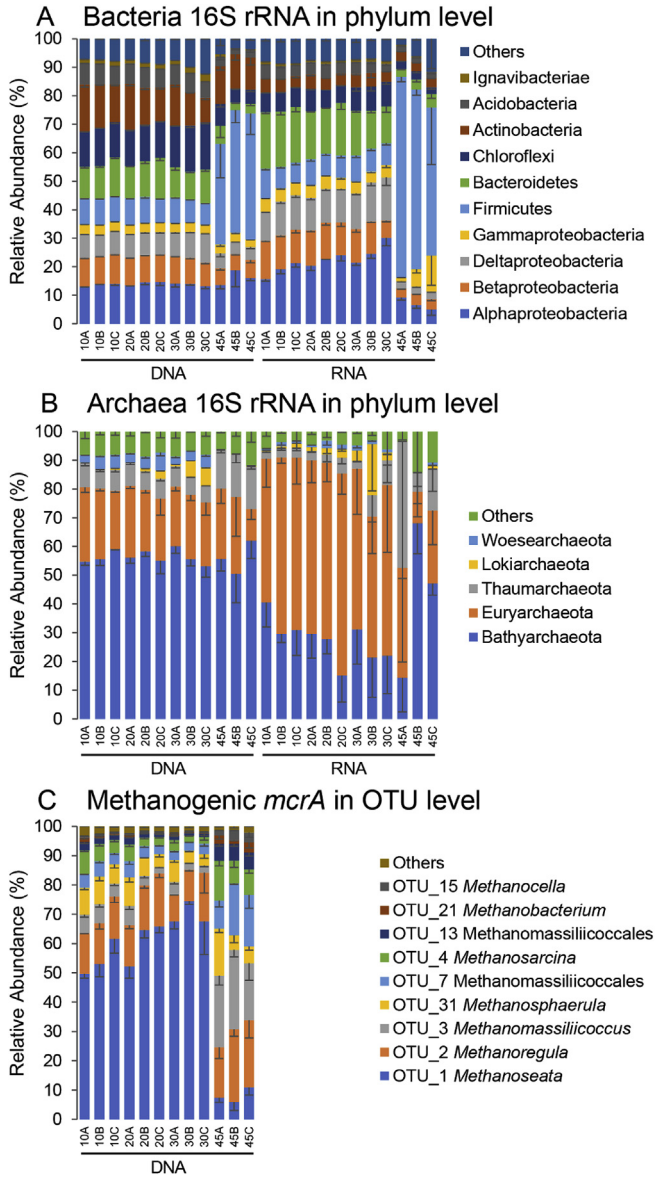

Fig. 5. Relative abundance of 16S rRNA bacteria, archaea and methanogens (Average relative abundance > 1%). The sample name, e.g. 10A, 10 represents temperature 10 °C and A represents incubation days of 25. B represents 63 days and C represents 100 days. Error bars indicate standard deviations of three replicates.

(45 °C), the methanogenic community composition was completely different, with *Methanomassiliicoccus* and *Methanoregula* being most abundant (Fig. 5C). NMDS analysis also showed a clear difference of the methanogenic community composition between samples at 45 °C and other temperatures (Fig. S4C).

4. Discussion

4.1. Functional response to temperature

The CH_4 production rate of incubated NamCo wetland soil (annual in situ temperature −0.6 °C) at 10 °C was 11% of that at 30 °C. In Zoige wetland soils (annual in situ temperature 2.2 °C), the rate at 10 °C was < 1% of the rate at 30 °C (Fu et al., 2015); and that rate was < 1% in temperate rice field soils (Lu et al., 2015) and ~4% in temperate peat soils (Freitag and Prosser, 2009). However, in arctic soils, the rate at 10 °C was ~50% of the rate at 30 °C (Tveit et al., 2015); and in subarctic peat soils, the rate at 10 °C was ~27% of that at 27 °C (Metje and Frenzel, 2007). These results show that, at low and moderate temperatures, CH_4 production in soils of NamCo wetland exhibited a lower temperature sensitivity than soils in Zoige wetland and some temperate ecosystems, but a higher temperature sensitivity than soils in arctic and subarctic ecosystems.

The linear increase of CH_4 with time at low and moderate temperatures (10, 20 and 30 °C) indicated that the microbes reacted rapidly to the temperature shift. Furthermore, increasing the temperature from 10 to 30 °C significantly increased CH_4 production, indicating that the optimum temperature of the soil microorganisms was much higher than the in-situ temperature. This is a common observation for aquatic environments (Schulz et al., 1997; Yao and Conrad, 2000; Yayanos, 1986).

At elevated temperature (45 °C), CH_4 production in NamCo soil was negligible and bacterial and archaeal ribosomal RNA decreased with incubation time, indicating inactivation or death of microbes. This is in contrast to many paddy soils, in which methanogenesis operates up to 50 °C (Yao and Conrad, 2000; Fey et al., 2001; Peng et al., 2008). However, CH_4 production in rice field soils collected in the Sanjiang Plain of Northeast China was also negligible at 45 °C unless supplemented with rice straw (Lu et al., 2015). Perhaps, samples from cold environments are more sensitive to exposure to elevated temperature than samples from temperate and tropical zones. However, this hypothesis requires more research. Alternatively, CH_4 production in NamCo soil at 45 °C may require prolonged incubation and perhaps addition of organic substrates to establish. It is noteworthy that copy numbers of *mcrA* genes were reasonably high (almost $10^8\ g^{-1}$ dry weight), only about one order of magnitude lower than at moderate temperatures.

The percentage of hydrogenotrophic methanogenesis was ~23% at low and moderate temperatures (10–30 °C). This percentage is close to the 33% that are theoretically expected and thus is consistent with the situation found in many different methanogenic environments (Conrad, 1999, 2002). Interestingly, the percentage of hydrogenotrophic methanogenesis was the same for all low and moderate temperatures (10–30 °C). This result is consisted with a study on a tidal freshwater sediment in which hydrogenotrophic methanogenesis was constant at ~25% at temperatures 8–32 °C (Avery and Martens, 1999). However, it is in contrast to Italian rice paddy soils, in which the hydrogenotrophic contribution slightly increased from 10 to 37 °C (Fey and Conrad, 2000).

In contrast to low and moderate temperatures, hydrogenotrophic methanogenesis dominated CH_4 production at 45 °C, albeit at a negligible rate. With such a shift from a mixture of acetoclastic and hydrogenotrophic methanogenesis to exclusively hydrogenotrophic methanogenenesis at the transition from moderate to thermophilic conditions, NamCo soils resembled the situation in many flooded soils (Fey et al., 2001; Peng et al., 2008; Conrad et al., 2009; Lu et al., 2015; Liu et al., 2018). The lack of acetoclastic methanogenesis at 45 °C was consistent with the accumulation of acetate and propionate and the decrease of pH at this temperature. By contrast, at temperatures of 10–30 °C, the fatty acids accumulated only in the presence of CH_3F, when acetoclastic methanogenesis was inhibited (Janssen and Frenzel, 1997). At 30 °C, where CH_3F was consumed and decreased from initially 1.8%–~0.4% at about 60 days of incubation, inhibition of acetoclastic methanogenesis was eventually released, as shown by increase of CH_4 production, consumption of acetate and increase of $\delta^{13}C$ values of acetate.

At 10 °C after about 50 days of incubation, the $\delta^{13}C$ values of acetate were significantly lower than those at higher temperatures. This observation indicates the operation of chemolithotrophic acetogenesis, which exhibits a stronger stable isotope fractionation than fermentative acetate production (Heuer et al., 2010; Blaser and Conrad, 2016). Chemolithotrophic H_2/CO_2-dependent acetogenesis is a more favored process at low than at moderate and high temperatures (Conrad et al., 1989; Nozhevnikova et al., 1994; Fu et al., 2018). The additional

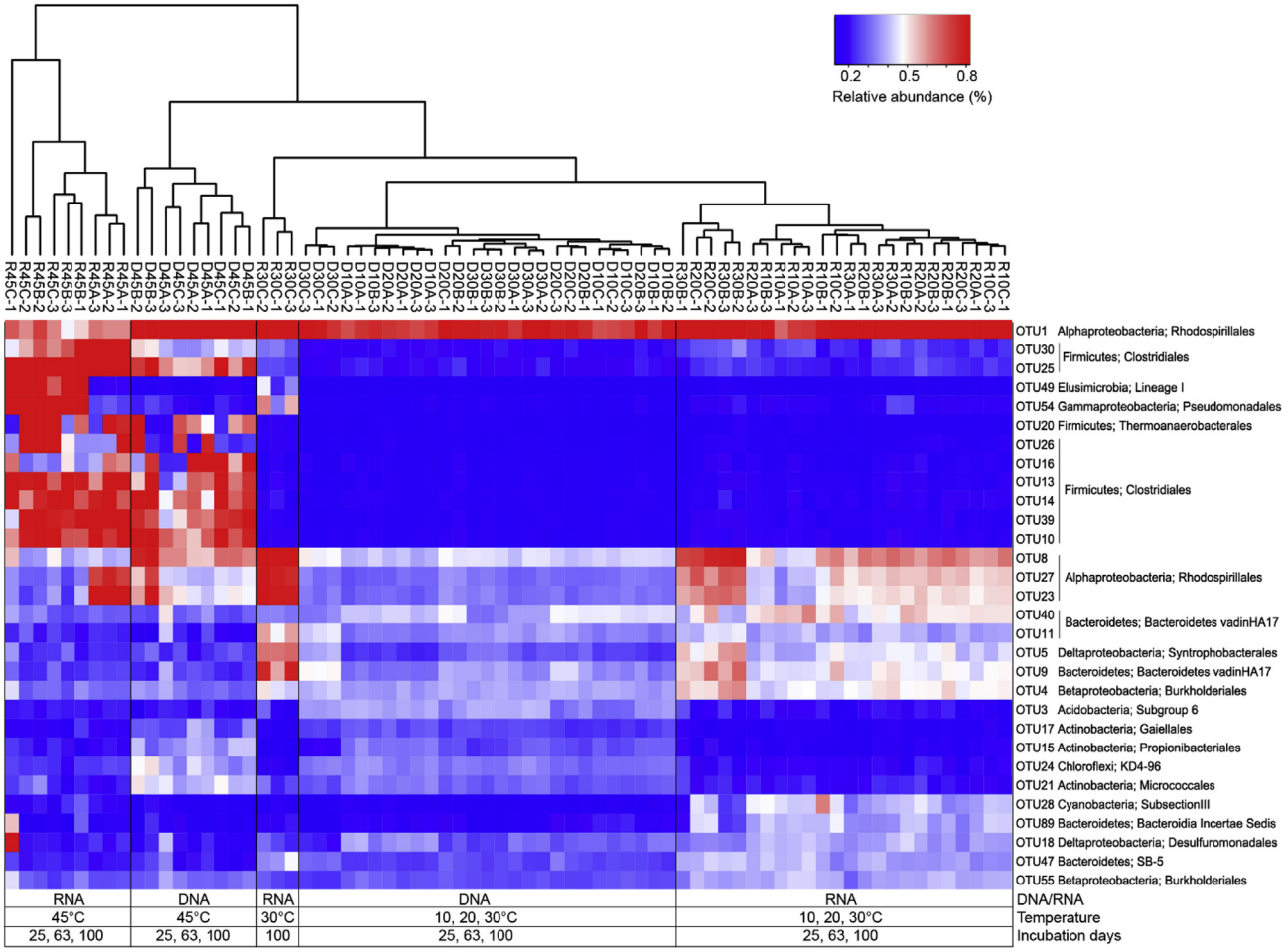

Fig. 6. 16S rRNA bacterial heatmap showing the relative abundance of selected OTUs. Manhattan distances was used to cluster the samples and OTUs. Red, white and blue color indicated by the color legend correspond to high, moderate and low relative abundance of the OTUs. The phylum and order information of each OTU is list in the right of the plot following the OTUs' name. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

acetate production may explain why the contribution of hydrogenotrophic methanogenesis is often less than the theoretically expected 33% (Conrad, 1999).

4.2. Microbial community response to temperature

In NamCo wetland soils, *Proteobacteria* was the dominant bacterial phylum at 10–30 °C. This is consisted with tropical lake sediments (Ji et al., 2016), Zoige peat soils (Cui et al., 2015), and Chinese wetlands in general (Zhang et al., 2018), but is different from arctic soils, where *Firmicutes* was the most abundant phylum and its relative abundance decreased with increasing temperature (Tveit et al., 2015). In NamCo wetland soil, *Firmicutes* (especially *Clostridia*) became the most abundant phylum only under thermophilic conditions at 45 °C. *Firmicutes* were also reported to be important in fermentation in high-temperature microbial mats (Klatt et al., 2013). *Clostridiales* were found being the dominant group for acetate turnover in rice field soils at 45 °C (Rui et al., 2009; Noll et al., 2010; Liu et al., 2018). Although we detected no significant consumption of acetate at 45 °C, putatively syntrophic acetate-oxidizing *Thermoanaerobacteraceae* (Liu and Conrad, 2010; Liu et al., 2018) were detected. Perhaps, these bacteria would become active after prolonged incubation at 45 °C and acetate consumption would resume.

The dominant methanogenic community is often quite different in different environments. In Finland peat soils, for example, the hydrogenotrophic order *Methanobacteriales* is dominant (Metje and Frenzel, 2005). In Amazonian lake sediments, the methanogenic taxa *Methanosaetaceae* (‘*Methanotrichaceae*’), *Methanomicrobiales*, *Methanobacteriales*, *Methanocellales* are all present (Ji et al., 2016). The same is true for various paddy soils all over the world (Scavino et al., 2013; Ramakrishnan et al., 2001; Reim et al., 2017; Zhang et al., 2018). However, soils in the Zoige wetlands have *Methanosarcinaceae* as the main methanogens (Fu et al., 2015). In NamCo wetland soils, at low and moderate temperatures (10–30 °C), acetoclastic *Methanosaetaceae* were the most abundant methanogens, followed by potentially hydrogenotrophic *Methanoregula* and *Methanosphaerula* (both *Methanomicrobiales*) and *Methanomassiliicoccus*. The latter are known to consume H_2 by reducing methanol (Paul et al., 2012). Since acetate was almost totally consumed in the NamCo wetland soils incubated at 10–30 °C, the high relative abundance of *Methanosaetaceae* was in good agreement with its higher affinity to acetate than *Methanosarcinaceae* (Jetten et al., 1990). The increase of relative abundance of *Methanosaetaceae* with increasing temperature was consist with studies in rice field soils (Fey and Conrad, 2000). The relative lower abundance of hydrogenotrophic methanogens compared to acetoclastic *Methanosaetaceae* is reasonable as hydrogenotrophic methanogenesis contributed comparatively little to total CH_4 production. Although temperature change between 10 and 30 °C affected the composition of the methanogenic community, the pathway of CH_4 production was not affected. This only happened at 45 °C.

In paddy soils under thermophilic conditions (45 °C), the order *Methanocellales* is commonly found (Conrad et al., 2009; Peng et al.,

2008; Fey et al., 2001). In our study, however, *Methanomassiliicoccales* and *Methanoregula* were the dominant clusters. *Methanomassiliicoccales* form a distinct cluster within the *Thermoplasmata* phylum and are distantly related to other methanogens (Paul et al., 2012; Dridi et al., 2012). *Methanoregula* is ubiquitous in wetlands and shows significant positive correlation with methane production capacity (Zhang et al., 2018). However, with extremely low abundance of methanogens and low CH_4 production detected at 45 °C, the activity of methanogenic groups at 45 °C was actually low and therefore, it is questionable which role *Methanomassiliicoccales* and *Methanoregula* played when NamCo wetland soil was incubated at 45 °C.

Bathyarchaeota, formerly named Miscellaneous Crenarchaeotal Group (MCG), are an uncultured archaeal phylum found in a wide range of environments (Meng et al., 2014; Zhou et al., 2018). Subgroups of them seem to be separated by saline and anoxic levels (Fillol et al., 2016) and encompass many different catabolic types (Zhou et al., 2018). In our anaerobic incubation of NamCo wetland soils, *Bathyarchaeota* were the dominant archaea at all temperatures, which indicated that they were not very sensitive to temperature change. Acetogenesis has been proposed for some lineages in the *Bathyarchaeota* phylum (He et al., 2016; Lazar et al., 2016) which means that during the incubation of NamCo wetland soils, acetate may have been produced not only by bacteria but also by *Bathyarchaeota*. Methylcoenzyme M reductase (MCR) is usually not found in microbes outside *Euryarchaeota*. Recently, however, MCR has been reported in the *Bathyarchaeota* phylum also implying that they may be involved in methanogenesis (Evans et al., 2015). However, *mcrA* gene sequencing did not provide evidence for *Bathyarchaeota* in our study, implying that putatively methanogenic *Bathyarchaeota* were rare or were not covered by the *mcrA* primers used in this study. However, *Bathyarchaeota* are also able to anaerobically utilize many different compounds (Zhou et al., 2018), including lignin (Yu et al., 2018). Hence it is presently difficult to assess the actual function of *Bathyarchaeota* in NamCo wetland soil, but at their high relative abundance, they should play a key role in the carbon cycle of NamCo wetland soil.

Woesearchaeota, formerly named Deep-sea Hydrothermal Vent Euryarchaeota Group 6 (DHVEG-6), was reported to have a potential syntrophic relationship with methanogens (Liu et al., 2018). In NamCo wetland soil, however, the relative abundance of *Woesearchaeota* was very low, and their relative abundance was even lower on RNA than DNA level, implying that they probably did not play an important role in NamCo wetland soil.

4.3. Microbial methanogenesis at alkaline pH

The methanogens in alkaline conditions were most investigated in saline alkaline soda lakes (Oremland et al., 1982; Antony et al., 2012; Sorokin et al., 2015a). The methanogens in these haloalkaline environments usually belong to the genera of *Methanocalculus*, *Methanosalsum* and *Methanohalophilus* (Liu et al., 1990; Zhilina et al., 2013; Sorokin et al., 2015b). However, we did not find these genera in alkaline NamCo wetland soils and there was no available reference of methanogenic communities in other alkaline wetland soils. Obviously, the methanogenic community structure and function in alkaline NamCo wetland soil had several characteristics that distinguished them from those in other acidic wetlands, like paddy soils or peatlands. However, the archaeal community resembled that of alkaline lake sediments from the Tibetan Plateau. When studying 20 different lake sediments (NamCo not included) with a range of pH between 6.9 and 10.4 and salinities between 0.3 and 42 g L^{-1}, Liu et al. (2016) found that the archaeal community composition was mainly affected by the salinity of the sediment. The methanogenic community of KongzhongCo sediment in the study of Liu et al. (2016) was most similar to that of NamCo wetland soil, notably the dominance of *Methanosaetaceae* followed by *Methanoregula*, together with a high relative abundance of *Bathyarchaeota* (formerly designated MCG). Similar to NamCo wetland soil, KongzhongCo sediment has a pH of 8.5–9.2 and a low salinity (1.8 g L^{-1}). Therefore, it is possible that the microbial methanogenic community of alkaline NamCo wetland soil and its functioning in CH_4 production is characteristic for alkaline soils and sediments.

5. Conclusion

The function of the methanogenic microbial community in alpine alkaline NamCo wetland soils was only partially similar to that of temperate neutral paddy soils. Notably, CH_4 was produced by acetoclastic and hydrogenotrophic methanogenesis in a ratio of about 3:1. However, acetoclastic methanogens were almost exclusively represented by the genus *Methanosaeta* that occurred at high relative abundance, while *Methanosarcina* played only a minor role. By contrast, the hydrogenotrophic methanogens were represented by *Methanoregula* and *Methanomassiliicoccus* rather than by *Methanocellales* or *Methanobacteriales*, which are prevalent in paddy soils. Similarly as in most methanogenic soils, acetoclastic methanogenesis ceased at 45 °C, but by contrast was not replaced by syntrophic acetate oxidation. Instead acetate accumulated under thermophilic conditions. It was most probably produced by fermenting *Firmicutes*, which dominated the bacterial community at 45 °C, while *Alphaproteobacteria* dominated at lower temperatures. A possible role of *Bathyarchaeota* in acetogenesis or methanogenesis is debatable. The observation that acetate consumption in NamCo wetland soils was impaired by incubation at 45 °C is reasonable, since the soils on the Tibetan plateau in contrast to those in more temperate climates will never reach such high temperature and therefore, many microbial groups will not be adapted. While hydrogenotrophic methanogenesis was still possible (albeit at low rates), acetoclastic methanogenesis and anaerobic syntrophic acetate oxidation were not.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.01.024.

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